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Introduction

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Body and Key Research Accomplishments

Task 1. Regulation of the p220 pathway in response to DNA damage. Zhao et al. found that p220 is indeed a target of DNA damage-induced pathways and displacement of p220 from Cajal bodies is associated with cell cycle arrest upon ionizing radiation-mediated DNA damage (Zhao 2004).

Task 2. Dissociation of Cajal bodies in response to loss of p220. We have shown that p220-deficient cells display defects in localization of a Cajal body marker, coilin (Ye et al. 2003). In order to answer the question whether this phenomenon represents a general disruption in Cajal body structure, we will immunostain $p220^{-/-}$ cells with a range of antibodies recognizing other Cajal body epitopes, such as SMN (*S*urvival of *M*otor *N*eurons) protein, which was previously proposed to be recruited to Cajal bodies by coilin (Hebert et al. 2001), and visualize Cajal bodies with high-resolution deconvolution microscopy. Our preliminary results indicate that SMN localizes to Cajal bodies and gems in p220-deficient cells (Nalepa and Harper, unpublished data). Therefore, it appears that the loss of $p220^{NPAT}$ displaces only some but not all components of Cajal bodies. This finding most likely reflects functional and structural heterogeneity of mammalian Cajal bodies, which parallels previous conclusions from the study of coilin knockout mice (Tucker et al. 2001), and argues that – in addition to coilin – other Cajal body proteins might be able to recruit SMN under some circumstances.

Task 3. To determine whether E7-mediated partial suppression of $p220^{-/-}$ phenotype is Rb-dependent. We have found that expression of human papillomavirus E7 protein allows p220-deficient cells to enter S-phase but does not rescue endogenous histone expression (Ye et al. 2003). It is well known that HPV E7 inactivates Rb, the critical regulator of G1/S passage encoded by retinoblastoma tumor suppressor gene , but it cannot be excluded that E7 might in parallel target another protein(s) to override cell cycle arrest in p220-null cells. To better understand the impact of the Rb tumor suppressor inactivation on p220-regulated S-phase entry pathway, we will use small hairpin-mediated siRNA to downregulate endogenous Rb expression in $p220^{flox/-}$ cells and employ BrdU staining and flow cytometry to determine whether loss of Rb is sufficient to promote S-phase entry in the absence of p220 function.

Task 4. To understand how p220 cooperates with HiNF-P in control of histone gene expression. It has previously been shown that p220 activates histone promoter-driven luciferase reporters in co-transfection assays (Ma et al. 2000; Zhao et al. 2000). We demonstrated for the first time that p220 specifically regulates endogenous histone expression while general level of transcription is not affected (Ye et al. 2003).Other laboratories have found that p220 physically interacts with HiNF-P (Mitra et al. 2003) as well as with other histone-specific transcription factors (Zheng et al. 2003) and therefore might serve as a co-activator in histone transcription

Task 5. Intranuclear mobility of p220 in response to cell cycle signals. Addressing this task has been hampered by the fact that overexpressed p220 tends to localize throughout nucleoplasm instead of being targeted to Cajal bodies only. Fluorescence recovery after photobleaching (FRAP) studies of EGFP-fused p220 will be performed upon generation of stable EGFP-p220-knock-in HCT cell line.

Conclusions

I have demonstrated that:

1. p220 is required for the viability of human somatic cells;
2. p220-negative cells are unable to enter S phase of the cell cycle;
3. p220 is required for endogenous histone transcription in both G1 and S phases of the cell cycle;
4. HPV E7 abrogates cell cycle arrest in p220-/- cells but does not rescue histone transcription in the absence of p220.

Our findings were published in December 2003 (Ye et al. 2003), and the essential function of p220 in the G1/S transition was confirmed by the siRNA approach (Gao et al. 2003). Since that time, the role of p220 in the regulation of mammalian cell cycle has been addressed by several research groups. It has been demonstrated that:

1. In response to DNA damage, p220 is dispersed from Cajal bodies and this coincides with inhibition of histone gene transcription. It has been know for a long time that genetic damage results in activation of DNA damage checkpoints, including the G1/S checkpoint, and now it appears that the shutdown of histone transcription is a component of this IR-induced G1 phase arrest (Su et al. 2004).

2. p220 physically interacts with OCA-S, a coactivator complex that plays essential role in activation of H2B transcription during S-phase (McKnight 2003; Zheng et al. 2003), and with HiNF-P, a transcription factor critical for transactivation of histone H4 genes (Mitra et al. 2003). These discoveries partially explain how p220 controls histone gene expression at a molecular level and also indicate why and how p220-positive Cajal bodies are tethered to the sites of histone transcription in cell cycle-dependent manner.

3. p220 dynamically interacts with the CBP/p300 histone acetyltransferase to promote S-phase entry (Wang et al. 2004).

Taken together, our work has allowed to demonstrate the critical role of $p220^{NPAT}$ and – from a wider perspective – cyclin E/Cdk2 in the control of mammalian cell cycle. Importantly, our study

References

Hebert MD, Szymczyk PW, Shpargel KB, Matera AG. 2001. Coilin forms the bridge between Cajal bodies and SMN, the spinal muscular atrophy protein. Genes Dev 15(20):2720-9.

Ma T, Van Tine BA, Wei Y, Garrett MD, Nelson D, Adams PD, Wang J, Qin J, Chow LT, Harper JW. 2000. Cell cycle-regulated phosphorylation of p220(NPAT) by cyclin E/Cdk2 in Cajal bodies promotes histone gene transcription. Genes Dev 14(18):2298-313.

Mitra P, Xie RL, Medina R, Hovhannisyan H, Zaidi SK, Wei Y, Harper JW, Stein JL, van Wijnen AJ, Stein GS. 2003. Identification of HiNF-P, a key activator of cell cycle-controlled histone H4 genes at the onset of S phase. Mol Cell Biol 23(22):8110-23.

Tucker KE, Berciano MT, Jacobs EY, LePage DF, Shpargel KB, Rossire JJ, Chan EK, Lafarga M, Conlon RA, Matera AG. 2001. Residual Cajal bodies in coilin knockout mice fail to recruit Sm snRNPs and SMN, the spinal muscular atrophy gene product. J Cell Biol 154(2):293-307.

Ye X, Wei Y, Nalepa G, Harper JW. 2003. The cyclin E/Cdk2 substrate p220(NPAT) is required for S-phase entry, histone gene expression, and Cajal body maintenance in human somatic cells. Mol Cell Biol 23(23):8586-600.

Zhao J. 2004. Coordination of DNA synthesis and histone gene expression during normal cell cycle progression and after DNA damage. Cell Cycle 3(6):695-7.

Zhao J, Kennedy BK, Lawrence BD, Barbie DA, Matera AG, Fletcher JA, Harlow E. 2000. NPAT links cyclin E-Cdk2 to the regulation of replication-dependent histone gene transcription. Genes Dev 14(18):2283-97.

Zheng L, Roeder RG, Luo Y. 2003. S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. Cell 114(2):255-66.

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The Cyclin E/Cdk2 Substrate p220^{NPAT} Is Required for S-Phase Entry, Histone Gene Expression, and Cajal Body Maintenance in Human Somatic Cells

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Cyclin E/Cdk2, a central regulator of the G_1/S transition, coordinates multiple cell cycle events, including DNA replication, centrosome duplication, and activation of the E2F transcriptional program. Recent studies suggest a role for cyclin E/Cdk2 in activation of histone transcription during S phase via the Cajal body-associated protein p220^{NPAT}, and in addition, p220 can promote S-phase entry independently of histone transcriptional activation when overexpressed. Here we have examined the requirement for p220 in histone transcription, cell cycle progression, and Cajal body function through analysis of human somatic HCT116 cells engineered to contain a conditional p220 allele. p220 is required for proliferation of HCT116 cells, as assessed after expression of Cre recombinase in p220$^{flox/-}$ cells. This defect was due to an inability of these cells to transit from G_0/G_1 into S phase, and cell cycle arrest occurred in the presence of elevated Cdk2 kinase activity. Expression of human papillomavirus E7, but not E6, eliminated cell cycle arrest in response to p220 depletion. Optimal expression of all four core histone genes required p220, as did optimal transcription of a histone H4 promoter-luciferase construct. Basal histone H4 expression in G_0/G_1, although p220 dependent, occurs in the absence of detectable phosphorylation of p220 on Cdk2 sites. Cells lacking p220 displayed defects in the localization of the Cajal body component p80^{coilin} as cells progressed from G_0 to S phase in response to mitogenic signals. These finding indicate that p220 is an essential downstream component of the cyclin E/Cdk2 signaling pathway and functions to coordinate multiple elements of the G_1/S transition.

The cell division cycle is a complex series of events that culminates in the duplication of the genome and segregation of replicated chromosomes into daughter cells. The decision to enter into the cell cycle in eukaryotes is made during G_1, a time when cells are poised to transduce growth factor signals that ultimately interface with the basic cell division machinery composed of cyclin-dependent kinases (Cdks). A central feature of the G_1 phase in normal diploid fibroblasts is the conversion of a cell from a growth factor-dependent proliferative cycle to growth factor-independent proliferation. In biochemical terms, this is thought to reflect the conversion of type D Cdk activity to type E Cdk activity (41, 42). Type D cyclins in complexes with Cdk4 serve as sensors of growth factor signaling and activate cyclin E/Cdk2 complexes through multiple mechanisms, including sequestration of the Cdk2 inhibitor p27 and activation of the E2F transcriptional program (reviewed in reference 42). Once cyclin E/Cdk2 is fully activated, cells are no longer dependent on stringent growth factor signals for further cell cycle progression. Indeed, previous experiments have demonstrated that activated cyclin E/Cdk2 complexes are sufficient to drive quiescent cells into S phase (8) and to promote S phase independently of the Rb/E2F pathway (22, 24).

Precisely how cyclin E/Cdk2 catalyzes S-phase entry is not clear, but its ability to coordinate multiple S-phase events is likely to be critical for genome integrity. S phase requires that multiple events occur concurrently, including activation of the DNA replication process, activation of centrosome duplication, and production of DNA synthesis precursors. Cyclin E has been implicated in each of these functions. However, relevant substrates for these activities are only beginning to be understood. Through inactivation of Rb and derepression of E2F1-3, cyclin E promotes the production of enzymes required for DNA replication and nucleotide synthesis (14, 35, 45). The critical nature of the cyclin E program is indicated by the fact that cells that persistently overexpress cyclin E display genomic instability (44, 33).

Proper DNA replication also requires that the core components of nucleosome, histones H2A, H2B, H3, and H4, are produced concurrently with S phase. Recent studies (34) have reinforced the established concept that DNA replication and histone gene expression synthesis are both functionally and temporally linked (21, 29). Loss of histone production leads to inhibition of DNA synthesis, independently of Cdk2 activity. Likewise, inhibition of DNA synthesis leads to repression of histone synthesis. Thus, it is well established that, during S phase, histone mRNA levels dramatically increase relative to those in other cell cycle phases, through both transcriptional and posttranscriptional mechanisms (11, 19; reviewed in reference 29). During S phase, histone mRNA is stabilized (about sevenfold) and processed via the action of SLBP1, which binds the 3′ stem-loop in histone mRNAs and facilitates processing via endonucleolytic cleavage, as well as histone translation (13, 40). SLBP1 accumulation is cell cycle regulated and increases

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during S phase through largely translational mechanisms (13, 51). Histone transcription is activated (about fivefold) concurrently with S phase, and this involves distinct transcriptional activators that function through subtype specific *cis*-acting elements in histone promoters (2, 3, 9, 10, 21, 28, 38, 47, 48, 49). Although activation of histone mRNA synthesis is clearly activated during S phase, it is not clear to what extent the expression of what are thought to be replication-dependent histone genes can occur outside S phase. However, previous studies have indicated detectable levels of histone mRNA in serum-deprived cell populations that contained 2 to 3% S-phase cells (11, 19). Whether this reflects incomplete cell cycle synchrony is an open issue.

Precisely how cell cycle positional information is relayed to the histone transcriptional apparatus is unknown. However, recent data implicate the cyclin E/Cdk2 substrate p220^{NPAT} as being involved in this process. p220 was identified as a cyclin E/Cdk2 binding protein (25, 52), and initial studies implicated p220 in control of the G_1/S transition and in histone gene transcription (26, 52, 53). p220 is localized in small nuclear organelles called Cajal bodies (CBs) (26, 53). CBs were first identified in 1903 (7), but their components and functions have only begun to emerge in the last decade (18, 31, 36). In normal diploid fibroblasts in G_1, two p220-containing CBs are associated with replication-dependent histone gene clusters on chromosome 6p21. As these cells enter S phase, two additional p220-positive CBs become associated with replication-dependent histone gene clusters on chromosome 1q21. p220-positive CBs persist until mitosis, when CB components become dispersed. By using phosphorylation-specific antibodies against Cdk2 sites in p220, we demonstrated that CB-associated p220 is phosphorylated during the cell cycle (26) and the timing of phosphorylation correlated with accumulation of cyclin E in CBs during late G_1 (23, 26). Consistent with a role in histone transcription, overexpression of p220 in tissue culture cells leads to activation of histone H4 and H2B promoter-reporter constructs through cell cycle regulatory elements in these promoters, and in this context, p220 is activated by cyclin E/Cdk2 (26, 53). It is not clear whether p220 is also required for activation of endogenous histone transcription.

Enforced expression of p220 drives cells from G_1 into S phase, apparently without affecting the ability of cells to progress through S phase (52). Recent work with p220 mutants that are unable to activate H4 transcription indicated that its ability to promote cell cycle entry is not dependent on its ability to activate histone transcription (50). Moreover, it is not clear whether p220 normally plays a direct role in allowing cells to enter S phase. To delineate the roles of p220 in cell cycle control, we have examined the phenotype of human somatic cells engineered to conditionally express p220. We found that p220 is essential for cell proliferation and that its absence during the G_1 phase of the cell cycle leads to cell cycle arrest prior to S-phase entry. This arrest occurs in the presence of high Cdk2 activity, suggesting that p220 activity is required downstream of Cdk2 activation. This arrest occurs concurrently with dissociation of the CB component p80^{coilin} from CBs, suggesting a role for p220 in maintaining proper CB assembly as cells attempt to enter the cell cycle. Interestingly, expression of human papillomavirus (HPV) E7—which inactivates pRb—overrides cell cycle arrest resulting from p220 depletion. In contrast, E6—which blocks p53 function—has no effect on S-phase entry in p220-null cells. p220-null cells entering S phase as a result of E7 expression display reduced levels of mRNAs for all four core histones, demonstrating a role for p220 in replication-dependent histone gene expression. p220 was also found to be critical for "basal" expression of histone H4 outside S phase. These data indicate that p220 is a central component in the cyclin E/Cdk2 signaling pathway responsible for orchestrating the G_1/S transition.

MATERIALS AND METHODS

Generation of targeting constructs. A human genomic library in λ phage was screened with the p220 cDNA, and one candidate clone (p220-22) containing exons 2 and 3 on a 10-kb genomic insert was identified. To generate a conditional knockout construct, the open reading frame of a neomycin (Neo) resistance gene linked with polyadenylation sequences (5) was inserted in frame into exon 2, and flanking *loxP* sites were inserted 613 nucleotides upstream and 734 nucleotides downstream of the Neo cassette. This was followed by insertion of an additional exon 2 and flanking intronic sequences, as well as an additional *loxP* site located 734 nucleotides 3′ to the duplicated exon 2. A conventional targeting construct contained a promoterless Neo cassette inserted in frame within exon 2, followed by 1.4 kb of exon 5 and flanking intronic sequences, which was produced by PCR from BAC clone RP11-56d3.

Generation of p220-deficient cells. HCT116 cells were transfected with a p220 conditional knockout construct with Lipofectamine, and selection was performed with medium containing G418. Screening for homologous recombination events at both 5′ and 3′ sequences was accomplished by PCR with Extended High Fidelity polymerase (Roche) and oligonucleotide pairs specific to the conditional allele and sequences outside the homology used for recombination (Fig. 1). One clone identified in this manner was infected with adenovirus (Ad)-Cre, and colonies were screened for loss of the exon 2-Neo fusion by PCR. These p220$^{flox/+}$ cells were then transfected with a promoterless Neo vector containing Neo in exon 2 and with exons 3 and 4 deleted. Recombination at the existing wild-type allele was verified by PCR analysis. PCR products representing the junctions of recombination sites were directly sequenced to verify their structures. To delete the conditional allele, p220$^{flox/-}$ cells were treated with Ad-Cre or Ad-LacZ as a control and cells were assayed as described below. Equal numbers of infectious particles of Ad-Cre and Ad-LacZ were used, as determined by immunostaining of infected cells with anti-Cre and by in situ β-galactosidase assays. To generate cells expressing an estrogen receptor-Cre fusion (ER-Cre), whose localization is under the control of hydroxyl-tamoxifen (OHT), p220$^{flox/-}$ cells or p220$^{+/+}$ cells as controls were transfected with a plasmid expressing ER-Cre recombinase linked with a puromycin resistance marker (32). Two days after transfection, cells were placed on selection medium containing 1 μg of puromycin per ml and three independent colonies were isolated for each genotype (p220$^{flox/-}$ ER-Cre and p220$^{+/+}$ ER-Cre). Immunoblotting of cell extracts from these cell lines indicated indistinguishable levels of ER-Cre, and immunofluorescence assay indicated cytoplasmic localization of ER-Cre in the absence of OHT and nuclear accumulation in the presence of OHT (data not shown). To generate E6 and E7 p220$^{flox/-}$ cells, p220$^{flox/-}$ cells were infected with retroviruses expressing either HPV E6 or E7. Twenty-four hours after infection, cells were placed on selection medium containing 1 μg of puromycin per ml and pooled puromycin-resistant cells were used in cell cycle and transcription experiments.

Cell synchronization and proliferation assays. For colony formation assays, p220$^{+/+}$, p220$^{flox/+}$, or p220$^{flox/-}$ cells were plated at a density of 100/100-mm-diameter culture dish prior to infection with either Ad-LacZ or Ad-Cre. Cells were incubated for 14 days, and the medium was replaced on alternate days. Colonies were visualized with methylene blue and prior to quantification. To examine colony formation in p220$^{flox/-}$ ER-Cre or p220$^{+/+}$ ER-Cre control cells, 200 cells were plated and cultured in the presence or absence of OHT and the number of surviving colonies was determined after 14 days with methylene blue. To examine cell proliferation, p220$^{flox/-}$ ER-Cre cells were cultured in the presence or absence of OHT. After 3 days, cells were trypsinized and counted. Ten thousand cells per 35-mm-diameter dish were plated for each group in triplicates in the presence or absence of OHT. At the indicated times, cells were released into 0.1% EDTA–phosphate-buffered saline and counted by trypan blue exclusion. For synchronization, p220$^{flox/-}$ or p220$^{+/+}$ control cells were infected with Ad-Cre or Ad-LacZ. Three days after infection, cells were starved with serum-free medium for 48 h and then stimulated to re-enter the cell cycle by

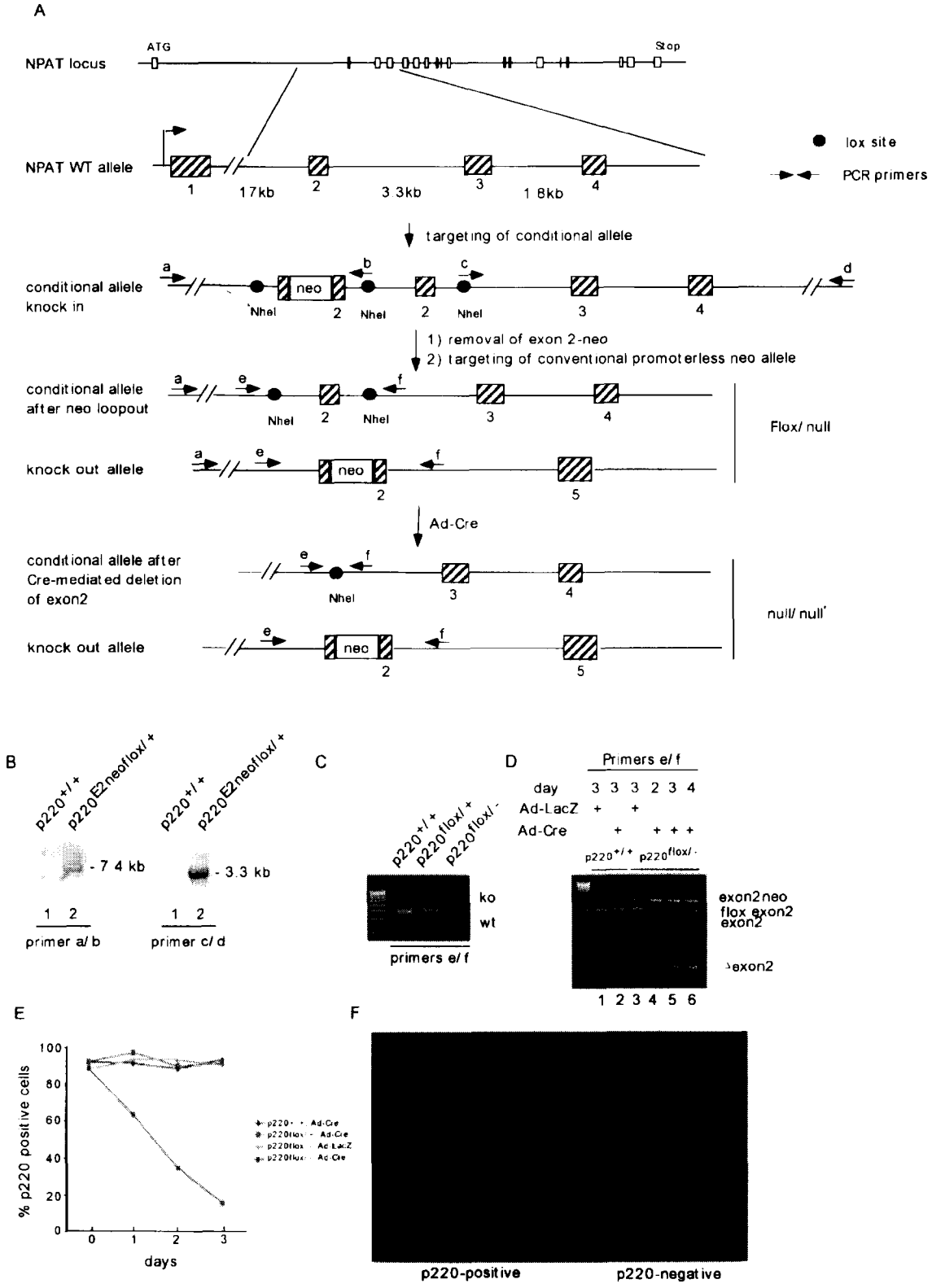


FIG. 1. Creation of HCT116 cells that conditionally express p220. (A) Structure of the p220 locus and approach used to generate $p220^{flox/-}$ cells. See the text for details of construction. Primers used for PCR are indicated. Primers b and c specifically recognize the fusion of *loxP* and p220 sequences and are therefore specific to the recombined allele. Primers a and d are outside the regions used for targeting. WT, wild type. (B) Homologous recombination of the conditional promoterless-*neo* targeting construct. Primers specific to the *loxP*-p220 junction (primers b and c) and primers outside the recombination boundary (primers a and d) were used in extended PCRs. Clone N118 contained the expected 7.4- and

addition of medium containing 10% serum for the indicated time. Cells were prepared either for immunofluorescence assay or for flow cytometry. In some experiments, aphidicolin (1 μg/ml) was used to block cells in S phase for 3 h.

Immunofluorescence assay and flow cytometry. For immunofluorescence assays, cells were grown on ultrathin cover slides (Fisher), fixed in 95% ethanol–5% glacial acetic acid, and permeabilized with 0.2% Triton X-100 for 10 min. Cells were then blocked with 5% goat serum (Sigma) for 30 min, incubated with primary antibodies (affinity-purified anti-p220 at 1:100) for 1 h, washed five times with phosphate-buffered saline, incubated with fluorochrome-conjugated secondary antibody for 1 h, and generously washed. For double immunostaining, cells at this point were fixed again and the immunostaining procedure was repeated with other primary and secondary antibodies. The following primary antibodies were used: anti-coilin monoclonal antibody (provided by M. Carmo-Fonseca, University of Portugal), anti-p220 DH4 monoclonal antibody (provided by J. Zhao, University of Rochester), and anti-phospho Pol II (Santa Cruz Biotechnology). To measure the relative levels of phosphorylated and unphosphorylated p220, cells were stained first with monoclonal antibody DH4 and subsequently stained with a antibodies directed against phosphor-T1350 (26). Nuclei were then counterstained with 4′,6′-diamidino-2-phenylindole (DAPI), and cells were mounted in SlowFade Light Antifade reagent (Molecular Probes). Images were taken on a Nikon/DeltaVision deconvolution microscope (Applied Precision) as a series of 0.2-μm-thick *z* sections, processed with a Softworx image workstation, and presented as a projection of transnuclear *z* sections, or on an Olympus BX60 microscope fitted with a Hamamatsu charge-coupled device camera.

For bromodeoxyuridine (BrdU) incorporation, cells were pulse-labeled with BrdU, incubated in 3 N HCl for 10 min, stained with AlexaFluor594-conjugated mouse anti-BrdU antibody (Molecular Probes), and then stained for p220 as described above. For bromouridine (BrU) incorporation, cells were pulse-labeled with BrU for 15 min, fixed, and stained with an anti-BrdU antibody. For flow cytometry, cells were harvested, fixed in ethanol, and stained with propidium iodide. Flow cytometry analysis was performed with a FACScan instrument.

Transcription analysis. For luciferase assays, p220$^{flox/-}$ ER-Cre or p220$^{+/+}$ ER-Cre cells at 70% confluence were rendered quiescent in the presence or absence of OHT and then transiently transfected in triplicate with pGL2-histone H4 promoter luciferase (53) and CMV-LacZ plasmids (0.1 μg) with Fugene 6 (Roche). Thirty-six hours after transfection, cells were collected and luciferase assays were performed with a luciferase assay kit (Promega) as previously described (50). β-Galactosidase assay were used to normalize the luciferase activities. To examine the expression of endogenous histone genes, mRNA was isolated from cells after synchronization, Ad infection, and release with RNAzol. mRNA was subjected to Northern blot analysis with histone H4 or β-actin as a probe or used for quantitative reverse transcription (RT)-PCR. mRNA was subjected to RT with oligonucleotides specific for each of the four core histones, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Quantitative PCR (in triplicate) was accomplished with SYBR-green fluorescence (Applied Biosystems) and an ABI Prism 7700 sequence detection system. The following oligonucleotides were used: H2A, 5′ primer GACGAGGAGCT CAACAAGCTG and 3′ primer TGTTGGGCAGGACACCG; H2B, 5′ primer CCGACACCGGCATCTCAT and 3′ primer GCGCTCGAAGATATCGTT GAC; H3, 5′ primer TACCAGAAGTCCACCGAGCTG and 3′ primer GATT TCTCGCACCAGGCG; H4, 5′ primer AATCCGCGATGCAGTTACCT and 3′ primer CCACGTCCATGGCTGTGA. For Northern blot assays, mRNA levels were quantified by phosphorimager analysis. The fold difference in histone transcripts versus histone transcript levels in p220$^{+/+}$ cells infected with Ad-LacZ was calculated by the ΔΔCT method, with GAPDH signals as an internal control.

Kinase assays. To examine Cdk2 kinase activity, the indicated cells were lysed in 50 mM Tris-HCl–2 mM EDTA–150 mM NaCl–0.1% Triton X-100–10 mM β-glycerol phosphate–5 mM NaF–10 mM *p*-nitrophenylphosphate–protease inhibitors (Roche) and cleared by centrifugation (14,000 × *g*, 15 min). Extracts (0.5 mg) were subjected to immunoprecipitation with anti-Cdk2 or control immunoglobulin G antibodies (Santa Cruz Biotechnology) in combination with protein A-agarose. Twenty percent of the washed immune complexes was used for histone H1 kinase activity as previously described (26). Reaction products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography. The remaining immune complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-Cdk2 antibodies to demonstrate equal Cdk2 levels.

RESULTS

Construction of human somatic cells that conditionally express p220^{NPAT}. In order to examine the role of p220 in cell cycle control, we used promoterless-*neo* technology (5) to generate human HCT116 cells that conditionally express p220. HCT116 cells are a well-characterized colorectal cancer cell line that has a stable karyotype and intact DNA damage and mitotic spindle checkpoints (6). Previous studies with random retroviral integration into the mouse genome had suggested that p220 is required for embryogenesis (12), and we therefore took into consideration the possibility that p220 might be required for cell proliferation and sought to make a conditional allele. The targeting construct for the conditional allele contained a duplication of exon 2 with the first exon 2 containing a promoterless neomycin resistance polyadenylation cassette (Fig. 1A). Both the E2-*neo* cassette and the duplicated exon 2 were flanked by *loxP* sites. Homologous targeting was established by PCR with primers that were specific to p220 sequences outside the targeting construct and primers that were composed of *loxP* and p220 sequences and were therefore specific to the conditional allele (Fig. 1B). Direct sequence analysis of these PCR products verified their identity (see Materials and Methods). The extent of homologous targeting was 1%. Following homologous targeting, the *neo*-disrupted exon was removed by Cre-mediated excision, leaving two *loxP* sites flanking an otherwise wild-type exon 2 sequence (p220$^{flox/+}$). A conventional targeting construct lacking exons 3 and 4 and containing a promoterless-*neo* cassette in exon 2 was then used to replace the remaining wild-type allele, generating p220$^{flox/-}$ cells (Fig. 1A and C).

To verify Cre-dependent excision of exon 2, p220$^{flox/-}$ cells were treated with Ad expressing Cre recombinase (Ad-Cre) or β-galactosidase (Ad-LacZ) and genomic DNA from these cells was examined for loss of the conditional allele by PCR (Fig. 1D). With Ad-Cre treatment, loss of the 1.5-kb PCR product derived from the floxed allele (but not the corresponding 2.6-kb product derived from the conventional null allele) was observed with concomitant appearance of the 200-bp product produced from the excised allele. In contrast, infection with Ad-LacZ did not alter either p220 allele (Fig. 1D). We verified loss of p220 expression with a highly sensitive immunofluorescence assay and deconvolution microscopy, which allows visu-

3.3-kb bands representing homologous recombination in 5′ (left side, lane 2) and 3′ (right side, lane 2) ends, respectively. These PCR products were absent in control HCT116 cells (left side, lane 1, and right side, lane 1). (C) PCR analysis of p220$^{flox/-}$ cells. Primers e and f (see panel A) were used for PCR with p220$^{+/+}$, p220$^{flox/+}$, and p220$^{flox/-}$ cells. The band at 1.5 kb represents either the wild-type (wt) or floxed allele, while the band at 2.6 kb represents the conventional promoterless-*neo* allele (ko). (D) Cre-mediated removal of floxed exon 2. p220$^{+/+}$ or p220$^{flox/-}$ cells were infected with Ad-LacZ or Ad-Cre for the indicated times, and genomic DNA was subjected to PCR analysis with primers e and f. Treatment of p220$^{flox/-}$ cells led to the formation of a 200-bp PCR product (lanes 4 to 6) representing deletion of exon 2 (Δexon 2). (E, F) Loss of p220 foci in p220$^{flox/-}$ cells expressing Cre recombinase. Asynchronous p220$^{+/+}$ or p220$^{flox/-}$ cells were treated with Ad-LacZ or Ad-Cre for the indicated time, and cells were subjected to immunostaining with anti-p220 antibodies (F). p220$^{flox/-}$ cells expressing Cre but not LacZ or p220$^{+/+}$ cells demonstrated progressive loss of p220 expression (E).

alization of p220 as brightly staining nuclear foci (26) (Fig. 1E and F). As early as 24 h after addition of Ad-Cre, ~40% of the cells lacked p220 staining (Fig. 1E and F). During the next 3 days, the percentage of p220-negative cells increased to ~85%. In contrast, Ad-LacZ-expressing cells or $p220^{+/+}$ cells expressing Cre retained p220 expression over the time course (Fig. 1E).

To facilitate analysis of $p220^{flox/-}$ cells, we also generated $p220^{flox/-}$ and corresponding $p220^{+/+}$ cells that constitutively express an ER-Cre fusion protein (32). ER-Cre is cytoplasmic in the absence of OHT but translocates to the nucleus upon its addition (32; data not shown). Relocation of ER-Cre to the nucleus leads to rapid removal of the conditional allele, as determined by genomic PCR analysis, and immunofluorescence assay of these cells demonstrated the expected loss of p220 protein (data not shown; see below).

$p220^{NPAT}$ is required for cell viability. We next examined whether p220 is required for viability in HCT116 cells. $p220^{flox/-}$ cells were treated with Ad-Cre (or Ad-LacZ as a negative control) for 2 days, which corresponded to the time when >60% of the cells displayed levels of p220 undetectable by immunofluorescence assay. Cells were then plated at low density for 12 days to allow colony visualization. $p220^{flox/-}$ and $p220^{flox/+}$ cells expressing LacZ formed colonies with high efficiency, while the colony formation seen with Ad-Cre was reduced by more than 10-fold (Fig. 2A and B). Given that a small fraction of the cells still expressed p220 on the basis of immunofluorescence assay results, we examined whether residual colonies contained an intact conditional p220 allele by genomic PCR. Of the more than 60 colonies examined in two independent experiments, none were found to have undergone excision of exon 2 (Fig. 2C and data not shown). Similar results were obtained with $p220^{flox/-}$ ER-Cre cells in the presence of OHT, which displayed an 80-fold reduction in colony number relative to that of cells in the absence of OHT (Fig. 2D and data not shown). Again, surviving colonies (n = 10) had not undergone excision of exon 2 (data not shown). In addition, $p220^{flox/-}$ ER-Cre cells grow poorly in the presence of OHT, relative to control $p220^{+/+}$ ER-Cre cells in the presence or absence of OHT (Fig. 2E). These data indicate that p220 is required for HCT116 cell viability and/or proliferation.

$p220^{NPAT}$ is required for cell cycle entry from G_0. The inability of $p220^{-/-}$ cells to proliferate led us to ask whether cell cycle arrest resulting from p220 depletion occurred at a particular stage. $p220^{flox/-}$ and control $p220^{+/+}$ cells were infected with either Ad-Cre or Ad-lacZ, and after 3 days, cells were deprived of serum for 48 h. Flow cytometry revealed substantial G_1 arrest under serum starvation conditions (11 to 28% of cells in S or G_2/M phase) (Fig. 3A and B). Serum was then added back to release the arrested cells into the cell cycle, and the ability of cells to enter S phase was assessed. At 13 h after serum addition, the percentage of S- and G_2/M-phase cells increased to >51% in $p220^{flox/-}$ cells expressing LacZ and $p220^{+/+}$ cells expressing Cre while only 21% of $p220^{flox/-}$ cells expressing Cre were in S or G_2/M phase (Fig. 3A and B). These data suggested that cells lacking p220 were defective in S-phase entry.

Because the efficiency of excision of the conditional p220 allele is less than 100%, we hypothesized that the small increase in S- and G_2/M-phase cells seen by flow cytometry in the presence of Cre reflected cells that had not undergone conversion to the $p220^{-/-}$ state. To examine this possibility, we labeled cells 13 h after release from quiescence with BrdU and then used an immunofluorescence assay to simultaneously score cells for BrdU incorporation and p220 expression (Fig. 3C). Overall, there was a 65% reduction in the number of BrdU-positive nuclei in $p220^{flox/-}$ cells treated with Ad-Cre, relative to cells treated with Ad-LacZ. The majority of Ad-LacZ-treated cells contained p220-positive foci, and of these p220-positive cells, 79% were BrdU positive (Fig. 3C), indicating efficient S-phase entry. In contrast, greater than 95% of Ad-Cre-treated cells lacking p220 foci were BrdU negative while 96% of the residual p220-positive cells in this culture were BrdU positive (Fig. 3C). These results indicate that p220 is required for S-phase entry from quiescence.

G_1 arrest in $p220^{-/-}$ cells occurs with high Cdk2 kinase activity. The inability of $p220^{-/-}$ cells to enter S phase could reflect a requirement for p220 downstream of cyclin E/Cdk2, or alternatively, loss of p220 could lead to activation of a checkpoint signal that leads to the inhibition of Cdk2 activity. Such inhibition could occur through induction of Cdk inhibitors, inhibitory phosphorylation of Cdk2, or degradation of cyclin A or E. To examine this question, we examined Cdk2 kinase activity in quiescent $p220^{flox/-}$ and $p220^{-/-}$ cells and in cells 13 h after serum-stimulated cell cycle re-entry. This corresponded to the time when more than 50% of LacZ-expressing $p220^{flox/-}$ cells have entered S phase (Fig. 3). As expected, Cdk2 kinase activity was low in quiescent cells and was increased at 13 h, reflecting cell cycle entry. Importantly, Cdk2 activity and protein levels were equivalent in $p220^{flox/-}$ cells expressing LacZ or Cre (Fig. 4, lanes 7 and 8) and were comparable to those seen in $p220^{+/+}$ cells treated similarly (Fig. 4, lanes 5 and 6). These data indicate that p220 is required for S-phase entry independently of cyclin E/Cdk2 and cyclin A/Cdk2 activity.

HPV E7 eliminates G_1 arrest in p220-null cells. Previous studies have highlighted a role for pRb and/or p53 in cell cycle arrest during the G_1 phase of the cell cycle. p53 is required for cell cycle arrest in G_1 in response to DNA damage via induction of the p21 Cdk inhibitor, while pRb is required for cell cycle arrest upon p21 induction (reviewed in references 41 and 42). pRb is also important for cell cycle arrest in response to differentiation and developmental signals. To examine the potential contribution of these G_1 checkpoint mechanisms to cell cycle arrest due to loss of p220, we used E6 and E7 proteins derived from HPV (4, 27, 39). E6 is known to functionally inhibit p53, while E7 blocks the activity of pRb. Pools of $p220^{flox/-}$ and $p220^{+/+}$ cells expressing either E6 or E7 were generated as described in Materials and Methods. Both $p220^{flox/-}$ E6 and $p220^{flox/-}$ E7 cells were found to undergo cell cycle arrest during serum deprivation in the presence of Ad-Cre. Cells were then stimulated to enter the cell cycle by serum addition and examined 13 h later by flow cytometry and BrdU incorporation (Fig. 5A and B). Upon serum stimulation, $p220^{flox/-}$ E6 cells displayed a 13.6% increase in S/G_2-M-phase cells, similar to that displayed by $p220^{flox/-}$ Ad-Cre-treated cells (Fig. 3A). In contrast, $p220^{flox/-}$ E7 cells displayed a larger increase in S/G_2-M-phase cells (33.2%), slightly smaller than that seen with $p220^{+/+}$ E7 cells (42.3%). When we examined them at the single-cell level by assaying BrdU incorpora-

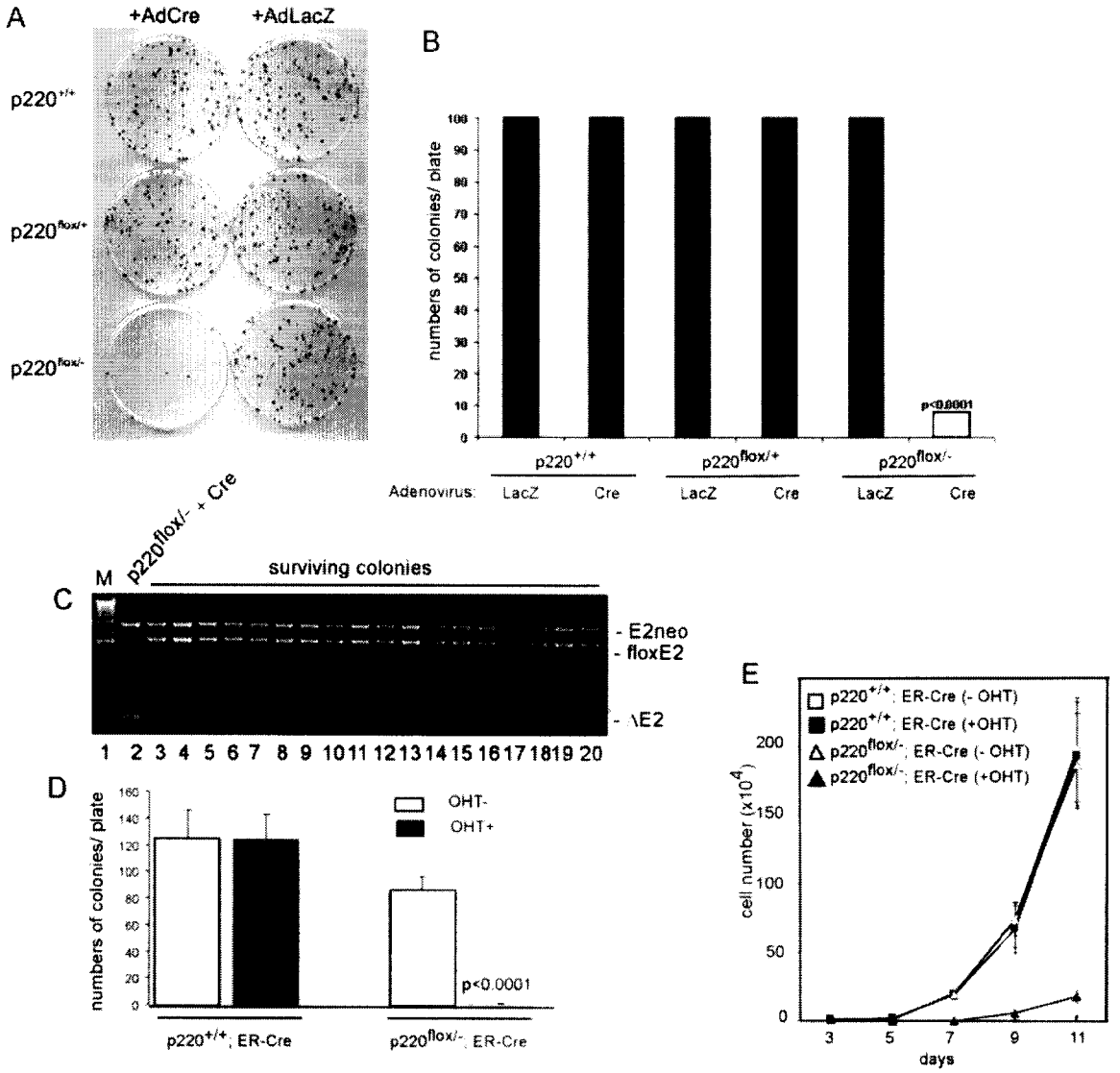
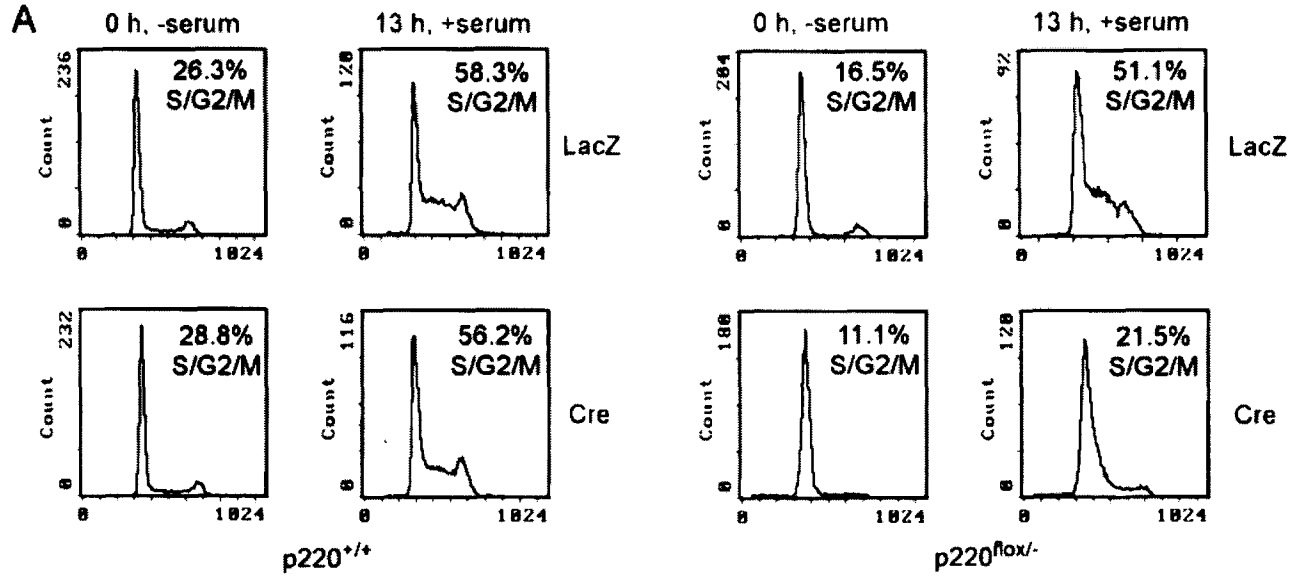


FIG. 2. p220 is required for cell viability. (A, B) Absence of colony formation in cells lacking p220. Cells of the indicated genotypes were infected with either Ad-LacZ or Ad-Cre for 2 days prior to plating at low density, in triplicate. Colonies were scored 2 weeks after plating. The average numbers of colonies formed are indicated in panel B. (C) Colonies surviving Cre recombinase contain an intact p220^{flox} allele. Genomic DNA was isolated from 18 residual surviving colonies and subjected to PCR with primers e and f (Fig. 1A) (lanes 3 to 20) along with DNA from p220$^{flox/-}$ cells transiently expressing Cre recombinase (lane 2). A total of 40 surviving colonies were tested and found to retain the conditional p220 allele. (D, E) p220$^{flox/-}$ ER-Cre cells are inviable in the presence of OHT. p220$^{flox/-}$ ER-Cre or p220$^{-/+}$ ER-Cre cells were grown for 2 days in the presence or absence of OHT, and 200 cells were plated in triplicate. After 2 weeks, surviving colonies were quantitated (D). To examine cell proliferation, cells were grown as in described for panel D and cells were plated equivalently at low density for each time point. At the indicated times, the number of viable cells was determined by trypan blue exclusion.

tion, we found that Ad-Cre-treated p220$^{flox/-}$ E6 cells displayed 15% BrdU labeling while p220$^{flox/-}$ E7 cells displayed 44% S-phase cells, comparable to the BrdU incorporation observed with Ad-Cre-treated p220$^{+/+}$ E6 (40%) and p220$^{+/+}$ E7 (64%) cells. As shown in Fig. 5B, p220-negative, BrdU-positive cells are readily seen in cultures of p220$^{flox/-}$ E7 cells after Ad-Cre treatment. These data indicate that E7 blocks a pathway responsible for mediating cell cycle arrest in response to p220 depletion. It is conceivable that pRb is a relevant target of E7 in this context.

p220^{NPAT} promotes histone gene expression. The abundance of core histone mRNA increases dramatically as cells enter S phase (10- to 35-fold in various experiments) (11, 19). This increase is due to both transcriptional and posttranscriptional mechanisms, although the transcriptional component is thought to represent only a three- to fivefold increase. The posttranscriptional accumulation of histone mRNA largely reflects the activity of SLBP1, which promotes histone mRNA processing during S phase, the cell cycle stage when it is most efficiently translated (reviewed in reference 29). It has been



B

genotype	virus	time (h)	Exp #1 G1	Exp #1 S/G2/M	Exp #2 G1	Exp #2 S/G2/M
$p220^{+/+}$	LacZ	0	72.6	26.3	74.8	22.6
	Cre	0	69.9	28.8	74.2	22.6
$p220^{flox/-}$	LacZ	0	81.5	16.5	77.4	19.3
	Cre	0	82.8	11.1	75.6	14.4
$p220^{+/+}$	LacZ	13	40.4	58.3	47.4	51.2
	Cre	13	41.9	56.2	47.9	50.6
$p220^{flox/-}$	LacZ	13	47.4	51.1	44.7	53.4
	Cre	13	78.5	21.5	72.8	23.5

FIG. 3. p220 is required for S-phase entry. (A, B) Defective S-phase entry in cells lacking p220. Cells of the indicated genotypes were infected with Ad-LacZ or Ad-Cre for 3 days, and cells were arrested in G_0 by serum deprivation for 2 days. Cells were either subjected to flow cytometry directly (−serum) or induced to enter the cell cycle by serum addition (13 h, +serum) prior to flow cytometry (A). The results of two independent experiments are shown in panel B. Experiment 1 corresponds to the data in panel A. (C) Absence of DNA replication in cells lacking p220. $p220^{flox/-}$ cells were synchronized in G_1 after infection with Ad-LacZ or Ad-Cre as described for panel A, and BrdU incorporation was examined 13 h after serum addition. Cells were subjected to a double-immunofluorescence assay with anti-BrdU and anti-p220 antibodies. Arrows indicate p220-deficient cells that are also BrdU negative. The asterisk indicates a residual p220-positive cell that is also BrdU positive.

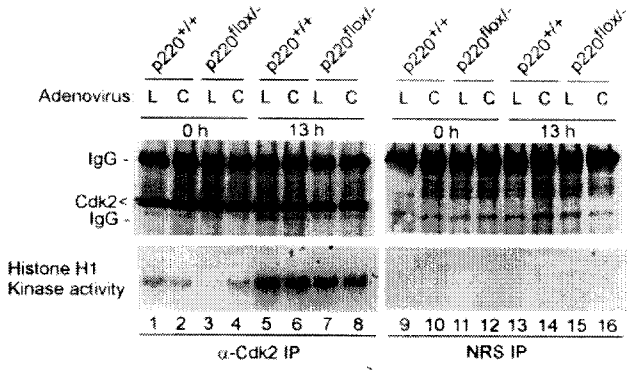


FIG. 4. Cell cycle arrest in p220$^{-/-}$ cells occurs in the presence of Cdk2 activity. Cells of the indicated genotypes were subjected to the synchronization procedure used for Fig. 3A in the presence of Ad-LacZ (L) or Ad-Cre (C). At the indicated times, cells were lysed and subjected to immunoprecipitation (IP) with normal rabbit immunoglobulin G (IgG) (NRS) or anti-Cdk2 antibodies. Washed immune complexes were subjected to immunoblotting with anti-Cdk2 antibodies or used in histone H1 kinase assays.

previously reported that p220 activates histone H4 and H2B reporter constructs in transient cotransfection experiments, implicating p220 in histone gene transcription (26, 53). This led us to ask whether cells deficient in p220 are defective in endogenous histone gene expression.

Initially, we examined histone H4 expression in cells that had been stimulated to re-enter the cell cycle from G_0 in the presence or absence of p220. As shown in Fig. 6A, p220$^{+/+}$ cells treated with Ad-Cre or Ad-LacZ and p220$^{flox/-}$ cells treated with Ad-LacZ displayed cell cycle-regulated expression of an ~400-bp histone H4 mRNA, as determined by Northern blot analysis of cells released from G_0. The level of histone H4 mRNA was low in G_0 and increased about sixfold as cells entered S phase (8 to 16 h) (Fig. 6A, lanes 1 to 12). In contrast, p220$^{flox/-}$ cells treated with Ad-Cre displayed substantially lower levels of histone H4 mRNA throughout the time course, increasing to a maximum of 1.7-fold at 13 h (Fig. 6A, lanes 13 to 16). The small increase in histone mRNA may reflect the absence of complete removal of the p220-floxed allele (Fig. 1). In order to quantify differences in expression and to expand the analysis to other histone subtypes, we used real-time RT-PCR in both G_0 cells and in cells released from quiescence by addition of serum (Fig. 6B and C). Subtype-specific primer sets were developed and found to give a single-peak dissociation curve, demonstrating PCR specificity (Fig. 6D and data not shown). In cells synchronized by serum deprivation, loss of p220 led to a 10-fold decrease in the abundance of histones H2A and H4, a 3-fold reduction in H3, and a 2-fold reduction in histone H2B mRNA, relative to controls with p220$^{+/+}$ cells infected with Ad-LacZ (Fig. 6B). Similar results were observed at 13 h (Fig. 6C), a time when histone H4 mRNA levels in p220-expressing cells was maximal (Fig. 6A).

We also used transient transfection of histone H4 promoter-reporter constructs to verify loss of histone transcriptional activity in cells lacking p220. Initially, we examined transcriptional activity in cycling p220$^{+/+}$ ER-Cre and p220$^{flox/-}$ ER-Cre cells. Cells were cultured for 36 h in the presence or absence of OHT and then transfected with a pGL-histone H4-luciferase reporter plasmid. Thirty-six hours later, cell extracts were assayed for luciferase activity. All three control groups (p220$^{+/+}$ ER-Cre cells with or without OHT and p220$^{flox/-}$ ER-Cre cells in the absence of OHT) displayed similar levels of luciferase activity. However, the level of activity observed in p220$^{flox/-}$ ER-Cre cells in the presence of OHT was substantially reduced in cycling cells (Fig. 6H). Similar experiments were performed with these cells after serum deprivation, which leads to substantial accumulation of G_1 cells. While the overall activity was reduced significantly, again loss of p220 expression (plus OHT) led to reduced levels of luciferase activity (Fig. 6H).

A major limitation of this approach for studying p220 function is that p220-null cells arrest in G_1 and therefore, we would anticipate that expression of replication-dependent histone genes would not occur in the absence of p220. In order to examine histone gene expression during S phase in the absence of p220, we took advantage of p220$^{flox/-}$ E7 cells, which enter S phase in the apparent absence of p220. With quantitative RT-PCR assays and cells released from serum starvation for 13 h, we found that the levels of expression for all four core histones were substantially reduced. The levels of H2A and H2B were reduced by 50% relative to those in p220$^{flox/-}$ E7 cells treated with Ad-LacZ, while the levels of H3 and H4 were reduced by more than fourfold (Fig. 5C). Again, the incomplete removal of p220 upon Cre treatment likely contributes to the overall level of histone transcription seen, and the actual contribution of p220 to histone transcription is likely to be larger. Taken together, these data indicate that p220 is critical for optimal levels of core histone expression as cells enter S phase.

Expression of replication-dependent histone genes outside S phase. In experiments examining histone gene expression in serum-starved cells, we noticed that histone H4 mRNA levels were also reduced in p220$^{-/-}$ cells, compared to those in p220-expressing cells (Fig. 6A and B). This, coupled with the fact that previous studies had seen a low, yet quantifiable, level of histone mRNA in G_0 and G_1 cells (11, 19), led us to ask whether p220 might contribute to low-level histone transcription outside S phase. In principle, the use of highly quantitative PCR techniques that were not available when early studies on histone transcription were performed might allow a more precise determination of whether "replication-dependent" histone expression can occur in the absence of DNA replication.

We first examined the possibility that the differences in the number of residual S-phase cells in these experiments due to p220 depletion is responsible for the differential histone expression observed in G_0/G_1 cell populations. However, we found that the percentage of residual BrdU-positive cells 48 h after serum deprivation was the same in both p220$^{flox/-}$ Ad-LacZ and p220$^{flox/-}$ Ad-Cre cells (~15%; data not shown). Thus, the difference in histone expression see in p220$^{flox/-}$ and p220$^{-/-}$ cells is unlikely to reflect the absence of complete synchrony. To examine histone expression outside S phase, we sought to further reduce the S-phase content of our synchronized population by depleting S-phase cells from the population with aphidicolin, an inhibitor of DNA replication. Previous studies have demonstrated that inhibition of replication by aphidicolin leads to a rapid depletion (<60 min) of histone mRNA. With a highly sensitive BrdU incorporation method,

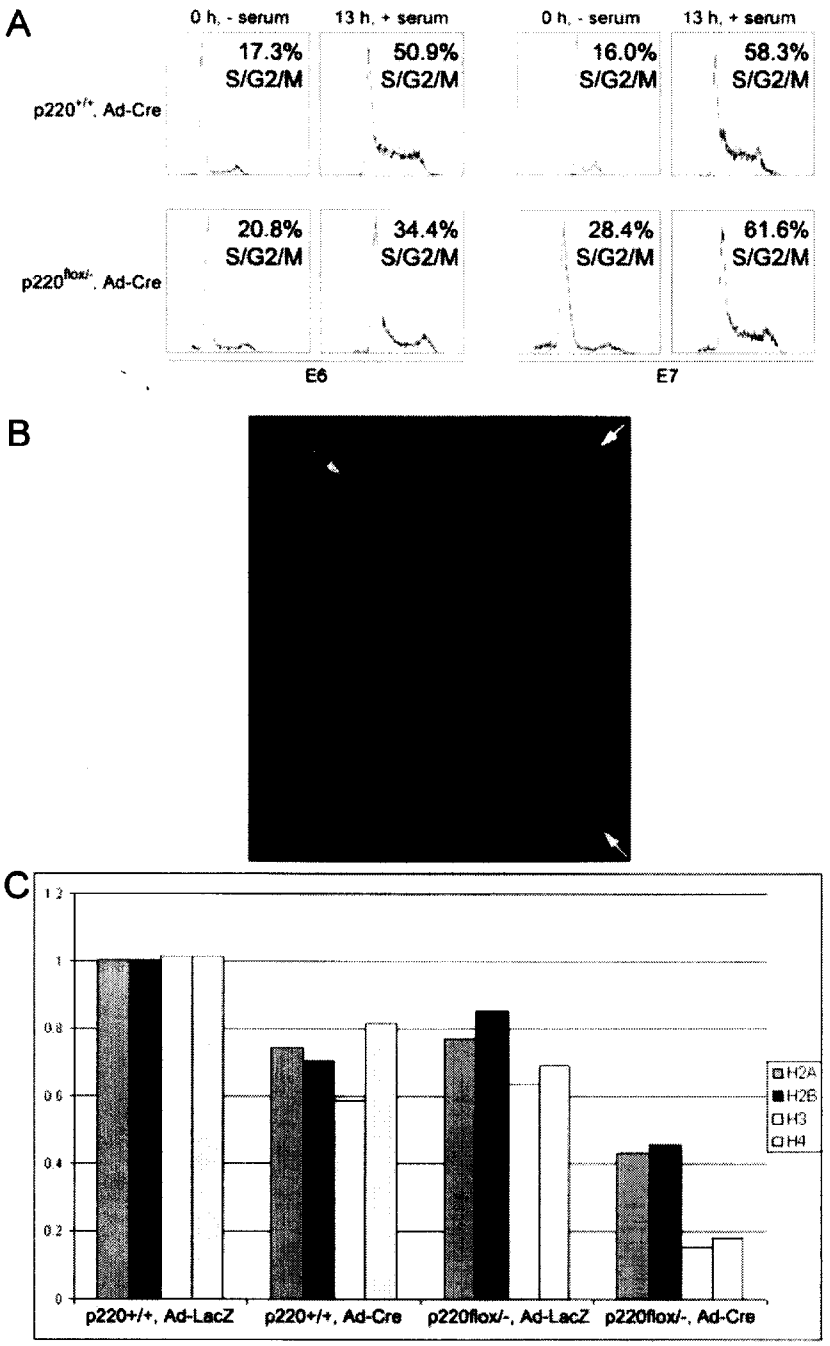


FIG. 5. HPV E7 eliminates G_1 cell cycle arrest in response to depletion of p220. (A) Expression of HPV E7, but not HPV E6, eliminates cell cycle arrest in G_1 in response to p220 depletion. Pools of $p220^{flox/-}$ or $p220^{+/+}$ cells expressing either E6 or E7 were arrested by serum starvation in the presence of Ad-Cre, and flow cytometry was performed before and after serum stimulation (13 h). (B) BrdU incorporation in p220-negative cells expressing E7. $p220^{flox/-}$ E7 cells were treated with Ad-Cre and subjected to serum starvation as described for Fig. 3. Cells were serum stimulated for 13 h, and pulse BrdU incorporation was examined by immunofluorescence assay together with p220 staining to identify cells that had not yet depleted p220. A BrdU cell still maintaining p220 expression is indicated by the green arrow, while cells undergoing DNA replication (red) while lacking detectable p220 staining (green) are indicated by the white arrows. (C) Reduced levels of replication-dependent histone gene expression in p220-null cells expressing E7. $p220^{+/+}$ E7 or $p220^{flox/-}$ E7 cells were treated with the indicated Ad, subjected to serum starvation for 48 h, and then stimulated to re-enter the cell cycle by serum addition. Thirteen hours later, the levels of histone mRNAs were determined by RT-PCR.

we were unable to detect any S-phase cells (n = 1,000) after 3 h in aphidicolin. Under these conditions, the levels of H2B and H4 mRNAs were reduced to 25 and 45%, respectively, compared with those in untreated cells (Fig. 6E). The BrdU conditions used coupled with deconvolution microscopy are sufficient to allow even a single replication focus to be visualized. Thus, there is clearly detectable H2B and H4 expression in the absence of detectable DNA replication. We then examined

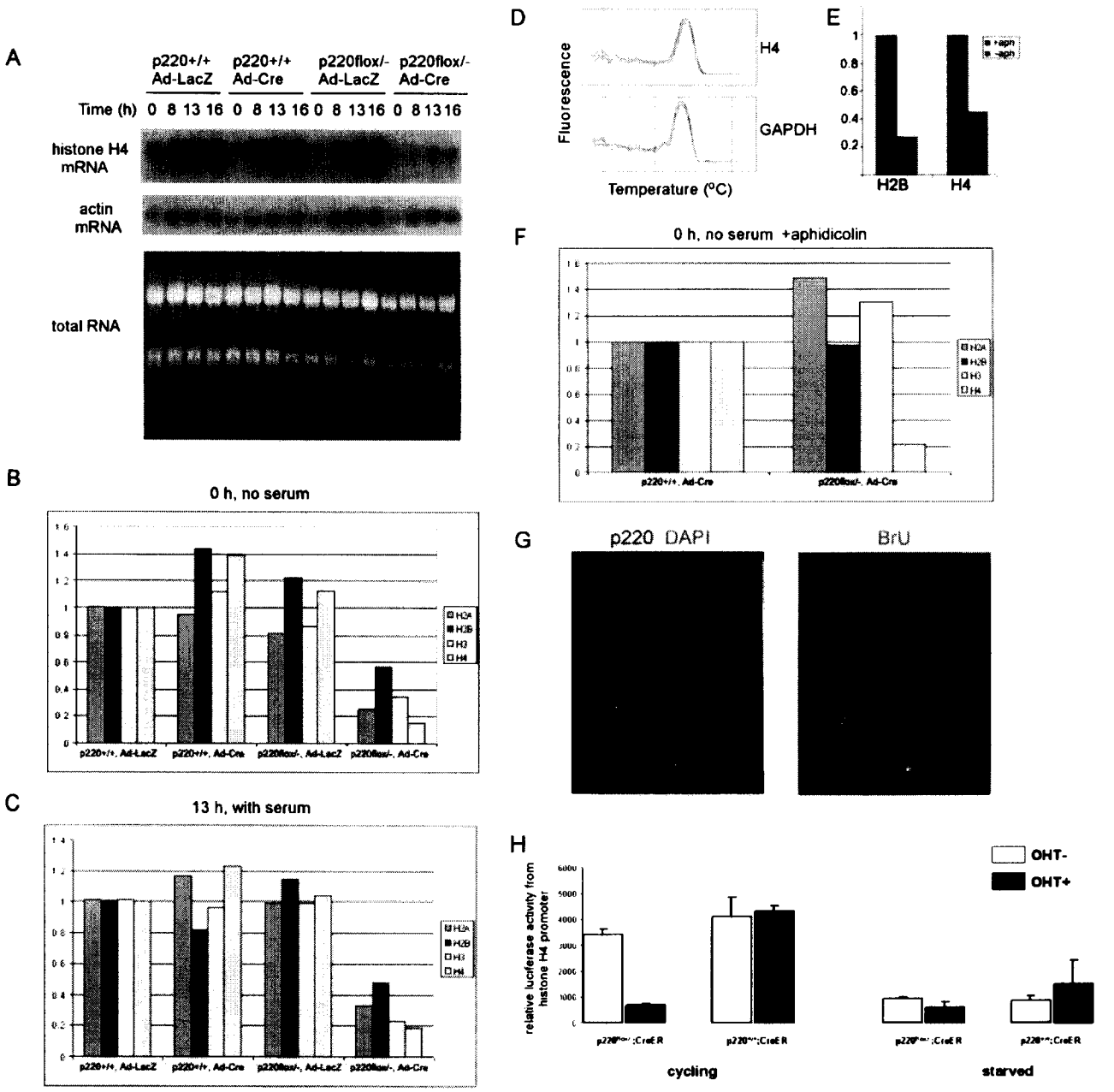


FIG. 6. p220 is required for histone gene expression. (A) Induction of histone H4 expression during S-phase entry is p220 dependent. Cells of the indicated genotypes were synchronized as described in the legend to Fig. 3A after treatment with Ad-Cre or Ad-LacZ. At the indicated times after serum addition, mRNA was isolated and used for Northern analysis with either a histone H4 or an actin probe. (B, C) p220 is required for optimal expression of all four core histone genes in G_0/G_1. mRNA from panel A was used for quantitative RT-PCRs with primers specific for histones H2A, H2B, H3, and H4, and GAPDH was used as a control (see Materials and Methods). Assays were performed in triplicate. (B) RNA from the 0-h time point was used. (C) RNA from the 13-h time point was used. (D) Dissociation curve analysis revealed a single peak for GAPDH and histone H4, indicating a unique RT-PCR product. Similar profiles were found for H2A, H2B, and H3 (data not shown). (E) Real-time quantitative PCR analysis reveals decreased but detectable levels of histone transcripts in HCT cells arrested in G_0/G_1 by serum deprivation and treated with aphidicolin (aph). Reactions were performed in triplicate. Black bars, RNA isolated from serum-starved $p220^{+/+}$ cells; red bars, RNA isolated from serum-starved $p220^{+/+}$ cells cultured in the presence of aphidicolin. (F) Loss of p220 results in decreased expression of histone H4 in serum-deprived, aphidicolin-treated cells. Real-time PCR assays were performed in triplicate. (G) p220-deficient cells are not defective in global transcription as determined by BrU incorporation. Asynchronous $p220^{flox/-}$ cells were treated with Ad-LacZ or Ad-Cre for 3 days, and cells were labeled with BrU for 0.25 h. Cells were subjected to p220 immunostaining, and BrU was then detected with an immunofluorescence assay. Representative p220-positive and -negative (white arrows) cells are shown. p220, red; BrU, green; DAPI, blue. (H) p220 is required for optimal histone H4 promoter reporter activity. $p220^{+/+}$ ER-Cre or $p220^{flox/-}$ ER-Cre cells were either allowed to cycle continuously or subjected to serum starvation in the presence or absence of OHT (as indicated) and then transfected with a pGL-histone H4-luciferase construct along with a plasmid expressing LacZ. After 36 h, cells were harvested and assayed for β-galactosidase and luciferase activities. Normalized luciferase activities from triplicate transfections, each assayed in triplicate, are shown.

whether this expression was dependent on p220. $p220^{flox/-}$ or $p220^{+/+}$ cells were serum starved and treated with Ad-Cre, and aphidicolin was added prior to mRNA isolation and quantitative PCR to examine the expression of histone genes. We found that the levels of histone H4 were dramatically reduced (fivefold) in $p220^{flox/-}$ cells treated with Ad-Cre relative to those in $p220^{+/+}$ cells treated similarly (Fig. 6F), indicating that p220 contributes to "basal" histone H4 transcription independently of DNA replication. In contrast, we were unable to detect significant differences in histone H3, H2A, and H2B levels under these conditions (Fig. 6F). The simplest interpretation of this finding is that HCT116 cells express what we refer to as basal levels of histone mRNAs outside S phase and that p220 contributes substantially to histone H4 expression independently of DNA synthesis in S phase.

Three lines of evidence indicate that the loss of histone mRNA is not a reflection of a general loss of transcription due to depletion of p220. First, β-actin levels accumulated in cells upon cell cycle re-entry independently of p220 status (Fig. 6A). Second, we examined global transcription by monitoring incorporation of BrU into newly transcribed mRNA with an immunofluorescence assay. The extent of BrU incorporation into $p220^{flox/-}$ cells transiently expressing Cre recombinase was indistinguishable in the presence or absence of p220, as determined by immunofluorescence assay (Fig. 6G). Third, we examined the intranuclear distribution of phosphorylated RNA polymerase II, which is an additional marker for ongoing transcription. Again, the punctate patterns of phosphorylated RNA polymerase staining were indistinguishable in $p220^{flox/-}$ and $p220^{-/-}$ cells (data not shown).

p220 phosphorylation status in G_0/G_1. The finding that p220 contributes to basal histone H4 expression outside S phase led us to examine the phosphorylation status of p220 in G_0/G_1 cells. Cyclin E/Cdk2 activity is frequently deregulated in tissue culture cells and could, in principle, lead to inappropriate p220 phosphorylation in G_0/G_1. This phosphorylation could, in turn, lead to increased histone expression outside S phase. HCT116 cells synchronized in G_0 by serum deprivation contain two p220 foci. However, these foci do not react with antibodies directed against phospho-T1350 (Fig. 7A). In contrast, HCT116 cells in S phase (13 h after release from serum deprivation) contain the expected four p220 foci and these foci react with the anti-phospho-T1350 antibodies (Fig. 7A). Similar results were obtained with antibodies directed against phospho-T1270 (data not shown). These data indicate that basal p220-dependent histone gene expression occurs without detectable phosphorylation of p220 on two prominent Cdk2 phosphorylation sites and suggest a phosphorylation-independent component to p220 function outside S phase.

$p220^{NPAT}$ deficiency leads to dissociation of $p80^{coilin}$ from CBs. CBs are complex organelles made up of a large number of proteins, many of which have not been identified (17, 18, 20, 36). A well-characterized CB component is $p80^{coilin}$. Although this protein is not essential for mouse development, its absence reduces association of the SMN protein with CBs (20, 46). We considered the possibility that p220 is required for proper localization of $p80^{coilin}$.

First, we examined $p80^{coilin}$ localization in asynchronous $p220^{flox/-}$ cells 5 days after infection with either Ad-Cre or Ad-lacZ (Fig. 7B). The vast majority of cells treated with Ad-LacZ maintained focal anti-p220 and anti-coilin staining (Fig. 7B). In contrast, cells treated with Ad-Cre demonstrated a dramatic increase in the number of cells displaying altered patterns of coilin staining. In particular, we noted cells that had lost focal coilin staining and exhibited either a diffuse pattern or a "dissociative" pattern containing a large number of small punctate foci (Fig. 7B). In this asynchronous population, 38% of p220-negative cells displayed dissociative or dispersed patterns of p220 staining. Similar results were obtained with $p220^{flox/-}$ ER-Cre cells treated with OHT (Fig. 7C and data not shown). We next examined coilin staining in synchronized cells lacking p220. $p220^{+/+}$ or $p220^{flox/-}$ cells were treated with Ad-Cre or Ad-lacZ and arrested in G_0 by serum withdrawal. Under these conditions, greater than 68% of the cells displayed normal patterns of coilin foci, independently of the presence of p220 (Fig. 7D). Previous studies have demonstrated that the number of coilin-positive CBs increases as cells enter S phase. When $p220^{flox/-}$ cells expressing Ad-LacZ were stimulated to re-enter the cell cycle with serum, the percentage of cells with focal anti-coilin staining increased to 79 to 92% and, as expected, a subset of coilin-positive foci in these cells were p220 positive. In contrast, with $p220^{flox/-}$ cells treated with Ad-Cre, less than 42% of these cells displayed focal coilin staining (Fig. 7D). These data indicate that p220 is not required for localization of coilin in CBs during G_0 but is important for efficient retention of coilin in CBs as cells traverse the G_1/S transition.

DISCUSSION

A central question in cell cycle control concerns the identity and functions of critical targets of cyclin-dependent kinases. Much of our understanding of the role of G_1 Cdks in cell cycle entry has come from analysis of Rb family members. In contrast, relatively little is known about cyclin E targets downstream of Rb. In this work, we have explored the function of p220, a cyclin E target implicated in G_1/S control. The notion that p220 is subject to regulation by cyclin E/Cdk2 is supported by several facts. First, cyclin E physically associates with p220 in vitro and in vivo (25, 52). Second, p220 is phosphorylated on at least five sites by cyclin E/Cdk2 in vitro and four of these have been detected in p220 isolated from mammalian cells with either mass spectrometry or phosphospecific antibodies (26). Third, coexpression of cyclin E/Cdk2 with p220 leads to stimulation of p220's ability to promote S-phase entry and histone gene transcription, while mutations in particular phosphorylation sites reduce histone reporter activation, suggesting a functional connection (26, 52, 53).

In this work, we sought to examine the extent to which cells use p220 to promote events linked with S-phase entry. With promoterless-*neo* technology, we generated HCT116 cells carrying a conditional allele of p220, allowing removal of p220 in response to expression of Cre recombinase. Several facts were established. First, p220 is required for HCT116 cell proliferation. Second, this defect in cell proliferation appears to manifest itself primarily in a block to DNA replication. G_0/G_1 cells lacking p220 are unable to enter S phase in response to serum, as determined either by flow cytometry or by BrdU incorporation assay. Third, this arrest occurs in the presence of S-phase levels of cyclin E/Cdk2 activity, confirming that p220

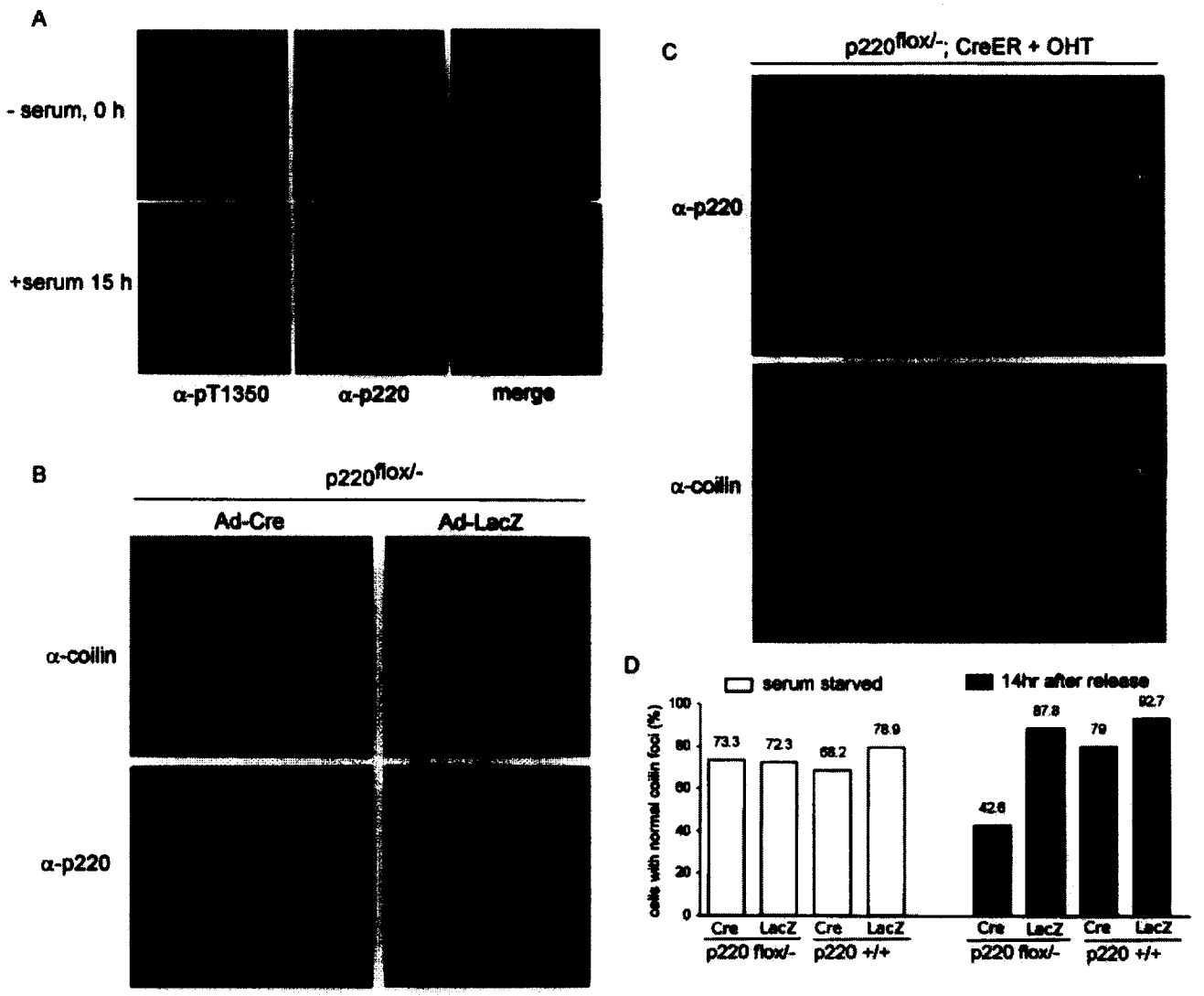


FIG. 7. p220 is unphosphorylated in G_0 HCT116 cells and is required for maintenance of p80^{coilin} localization during the G_1/S transition. (A) p220 is not phosphorylated on Thr-1350 in G_0 HCT116 cells. HCT116 cells were rendered quiescent by serum deprivation (2 days) and either maintained in the absence of serum (0 h) or induced to enter the cell cycle by serum addition (15 h). Cells were subjected to immunofluorescence assays with rabbit antibodies directed against either phospho-T1350 in p220 (green) or a monoclonal antibody directed against p220 (53) (red). Nuclei were stained with DAPI (blue). Cells in G_0 (no serum) contain two p220-positive foci, while cells in S phase (15 h) contain four p220 foci. p220-positive foci react with anti-T1350 in S-phase cells but not in G_0 cells. (B to D) Altered p80^{coilin} localization in cells lacking p220. (B) Asynchronous p220$^{flox/-}$ cells were treated with Ad-LacZ or Ad-Cre, and after 5 days, cells were subjected to immunofluorescence assays with anti-p80^{coilin} and anti-p220 antibodies: green, p80^{coilin}; red, p220; blue, DAPI. Some cells displayed altered p80^{coilin} localization typified by a dissociative staining pattern (arrow). (C) Asynchronous p220$^{flox/-}$ ER-Cre cells were grown in the presence or absence of OHT (4 days). p80^{coilin} and p220 proteins were detected by immunofluorescence assay as for panel B. (D) Disruption of p80^{coilin} localization during the G_1/S transition. Cells of the indicated genotypes were infected with Ad-Cre or Ad-LacZ for 3 days, serum was removed for 2 days, and then cells were either maintained in low-serum medium (0 h) or induced to reenter the cell cycle by serum addition (13 h). Cells were subjected to immunofluorescence assay as described for panel B, and cells with focal, dissociative, and dispersed p80^{coilin} were determined along with the status of p220 staining. At least 100 cells per group were counted.

functions downstream of cyclin E/Cdk2 activation. Fourth, expression of HPV E7, but not E6, allows S-phase entry in the absence of p220, implying a role for pRb in maintaining a G_1 block when p220 levels fall below a crucial level. Fifth, optimal expression of histone genes during S phase requires p220. Sixth, low levels of histone gene expression were detected outside S phase and expression of histone H4 in this setting was dependent on p220 but apparently independent of p220 phosphorylation by Cdk2. Finally, p220 is required to maintain focal localization of p80^{coilin} in CBs as cells traverse G_1/S.

The available data suggest that p220 plays multiple independent roles in cell cycle progression (Fig. 8A). We previously showed that mutations in p220 that block its ability to activate transcription of histone reporter constructs have little effect on the ability of p220 to promote S-phase entry (50). Thus, it seems likely that p220's histone transcriptional activation func-

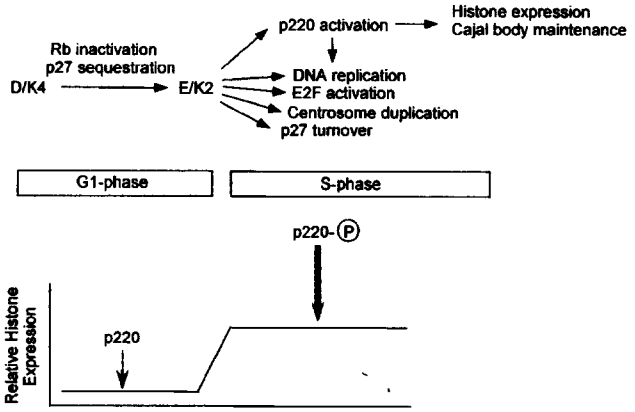


FIG. 8. p220 functions downstream of cyclin E/Cdk2 to control DNA replication and histone gene transcription. (Top) The cyclin E/Cdk2 signaling pathway. See text for details. (Bottom) Model for control of histone transcription by p220. During the G_0 and G_1 cell cycle phases, histone transcription is low but detectable. In this cell cycle phase, expression of histone H4 appears to be under the control of p220, although p220 is not phosphorylated by Cdk2. As cells enter S phase, p220 is phosphorylated by Cdk2 and this leads to an increase in p220's ability to promote expression of all four core histone genes.

tion is not critical to its S-phase-promoting function. This hypothesis is corroborated by our finding that inactivation of pRb by E7 rescues S-phase entry but not core histone expression in p220-deficient cells. E7 promotes S-phase entry by multiple mechanisms that include (27), but are not limited to (39), derepression of Rb-suppressed transactivation of cyclin E. Thus, expression of E7 shifts the cell cycle balance from Rb-mediated G_1 arrest toward constitutive activation of cyclin E/Cdk and accelerates progression through the G_1/S transition. This reduction of the S-phase entry threshold might be sufficient to promote DNA replication but not full histone transcription in the absence of p220.

Since core histones are essential for cell survival and their evolutionary conservation is extremely high, we believe that replication of DNA in the absence of proper synthesis of core histones might lead to aberrant chromatin assembly and organization. This, in the long term, would possibly result in cellular lethality. More importantly, the cell cycle-related phenotype of E7 $p220^{-/-}$ cells provides another compelling piece of evidence that $p220^{NPAT}$—in concordance with previous research (50)—controls multiple cellular pathways at the G_1/S boundary. As shown here, these pathways can be functionally separated because only DNA replication, but not histone transcription, is restored by inactivation of Rb in a $p220^{-/-}$ background. However, the mechanistic details of how p220 promotes DNA replication and histone transcription remain to be elucidated.

The majority of studies on histone transcription have focused on S-phase-dependent activation, and little is known about the pathways controlling basal histone transcription that occurs in G_0 or G_1. There are both common and specific *cis*-acting regulatory elements in replication-dependent histone genes (21). Both subtype-specific consensus elements and the common YY1 element located in the structural gene contribute to cell cycle-regulated transcription, and in some cases, *trans*-acting factors that interact with subtype-specific consensus elements have been identified (Oct1 in the case of H2B and IFR2 in the case of histone H4) (3, 9, 10, 15, 28, 47–49). Our work has uncovered what appears to be a phosphorylation-independent function of p220 in histone H4 expression, but not expression of other core histones, outside S phase. We propose (Fig. 7B) that during G_0/G_1, unphosphorylated p220 functions to support low levels of histone H4 expression. As cells approach the G_1/S transition and p220 becomes phosphorylated by Cdk2 (26), p220 activity is enhanced, leading to an approximately fivefold increase in core histone gene expression in HCT116 cells. Replication-dependent histone genes are relatively unique in that they are organized in large clusters of genes on chromosomes 1 (~13 genes) and 6 (~55 genes) (1, 30). Presumably, this organization is important for the coordinate expression of the four core histone subtypes, such that a balance of histone precursors is generated during S phase. The tethering of p220-containing CBs with these gene clusters is interesting in this regard and presumably indicates local action of p220 in promoting coordinate expression of these genes. It remains to be determined whether the pathways used by p220 to activate histone H4 transcription during G_0/G_1 are analogous to those used to achieve higher levels of transcription during S phase and precisely how p220 activates transcription of all for classes of histone genes. One possibility is that p220 functions through elements common to all core histone genes, thereby providing a mechanism for coordinate activation of these genes. It is also possible that p220 plays additional roles in histone mRNA accumulation, possibly through effects on posttranscriptional histone mRNA processing. The identification of proteins that interact with p220 is required to more fully understand how p220 functions to control histone transcription and S-phase entry.

Our work has also revealed a role for p220 in maintaining focal $p80^{coilin}$ localization as cells enter S phase. CBs are heterogeneous in nature (reviewed in reference 36). In the HCT116 cells used here, p220-positive foci always contain $p80^{coilin}$ but not all $p80^{coilin}$-containing foci, CBs, contain p220. The basis of this heterogeneity is unknown, but it likely reflects the fact that p220-positive CBs are tethered to histone gene clusters while other CBs are bound to other gene loci, including snRNA genes (16, 43). Previous studies have demonstrated that the number of CBs and the intensity of $p80^{coilin}$ immunostaining increase as cells progress from G_1 into S phase. This is due, in part, to the generation of two p220-containing CBs tethered to chromosome 1 histone gene clusters during S phase (26, 53). In HCT116 cells arrested by serum deprivation, the focal nature of localization is maintained in the absence of p220. When $p220^{flox/-}$ cells are induced to enter the cell cycle from G_0, focal $p80^{coilin}$ staining is maintained, as expected. In contrast, cells lacking p220 display aberrant patterns of coilin staining. In particular, a significant fraction of cells display what we refer to as dissociative $p80^{coilin}$ localization. This pattern features punctate $p80^{coilin}$ staining throughout the nucleus. We found that a subset of p220-deficient cells maintained $p80^{coilin}$ staining. Given the finding that a substantial fraction of quiescent HCT116 cells failed to enter S phase by 13 h after serum addition (Fig. 3A), it is possible that p220-negative cells that retain focal $p80^{coilin}$ staining represent those that did not exit G_0. We postulate that the presence of p220 in

CBs is required to maintain focal p80^{coilin} staining. This suggests a dynamic interplay among p220, p80^{coilin}, and CBs. These organelles are known to be highly dynamic and to undergo ATP-dependent reorganization (37). The mechanism by which p220 promotes p80^{coilin} localization remains to be determined.

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Y.W. and G. N. contributed equally to this work.

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REFERENCES

1. **Albig, W., and D. Doenecke.** 1997. The human histone gene cluster at the D6S105 locus. Hum. Genet. **101:**284–294.
2. **Aziz, F., A. J. van Wijnen, J. L. Stein, and G. S. Stein.** 1998. HiNF-D (CDP-cut/CDC2/cyclin A/pRB-complex) influences the timing of IRF-2-dependent cell cycle activation of human histone H4 gene transcription at the G_1/S phase transition. J. Cell. Physiol. **177:**453–464.
3. **Aziz, F., A. J. van Wijnen, P. S. Vaughan, S. Wu, A. R. Shakoori, J. B. Lian, K. J. Soprano, J. L. Stein, and G. S. Stein.** 1998. The integrated activities of IRF-2 (HiNF-M), CDP/cut (HiNF-D) and H4TF-2(HiNF-P) regulate transcription of a cell cycle controlled human histone H4 gene: mechanistic differences between distinct H4 genes. Mol. Biol. Rep. **25:**1–12.
4. **Berezutskaya, E., and S. Bagchi.** 1997. The human papillomavirus E7 oncoprotein functionally interacts with the S4 subunit of the 26S proteasome. J. Biol. Chem. **272:**30135–30140.
5. **Brown, J. P., W. Wei, and J. M. Sedivy.** 1997. Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. Science **277:**831–834.
6. **Bunz, F., A. Dutriaux, C. Lingauer, T. Waldman, S. Zhou, J. P. Brown, J. M. Sedivy, K. W. Kinzler, and B. Vogelstein.** 1998. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. Science **282:**1497–1501.
7. **Cajal, S. R. Y.** 1903. Un sencillo metodo de coloracion seletiva del reticulo protoplasmatico y sus efectos en los diversos organos nerviosos de vertebrados e invertebrados. Trab. Lab. Investig. Biol. **2:**129–221.
8. **Connell-Crowley, L., S. J. Elledge, and J. W. Harper.** 1998. G_1 cyclin-dependent kinases are sufficient to initiate DNA synthesis in quiescent human fibroblasts. Curr. Biol. **8:**65–68.
9. **Dailey, L., S. M. Hanly, R. G. Roeder, and N. Heintz.** 1986. Distinct transcription factors bind specifically to two regions of the human histone H4 promoter. Proc. Natl. Acad. Sci. USA **83:**7241–7245.
10. **Dailey, L., S. B. Roberts, and N. Heintz.** 1988. Purification of the human histone H4 gene-specific transcription factors H4TF-1 and H4TF-2. Genes Dev. **2:**1700–1712.
11. **DeLisle, A. J., R. A. Graves, W. F. Marzluff, and L. F. Johnson.** 1983. Regulation of histone mRNA production and stability in serum-stimulated mouse 3T6 fibroblasts. Mol. Cell. Biol. **3:**1920–1929.
12. **Di Fruscio, M., H. Weiher, B. C. Vanderhyden, T. Imai, T. Shiomi, T.-A. Hori, R. Jaenisch, and D. A. Gray.** 1997. Proviral inactivation of the *Npat* gene of Mpv 20 mice results in early embryonic arrest. Mol. Cell. Biol. **17:**4080–4086.
13. **Dominski, Z., L. X. Zheng, R. Sanchez, and W. F. Marzluff.** 1999. Stem-loop binding protein facilitates 3′-end formation by stabilizing U7 snRNP binding to histone pre-mRNA. Mol. Cell. Biol. **19:**3561–3570.
14. **Dyson, N.** 1998. The regulation of E2F by pRb-family proteins. Genes Dev. **12:**2245–2262.
15. **Eliassen, K. A., A. Baldwin, E. M. Sikorski, and M. M. Hurt.** 1998. Role for a YY1-binding element in replication-dependent mouse histone gene expression. Mol. Cell. Biol. **18:**7106–7118.
16. **Frey, M. R., and A. G. Matera.** 1995. Coiled bodies contain U7 small nuclear RNA and associate with specific DNA sequences in interphase human cells. Proc. Natl. Acad. Sci. USA **92:**5915–5919.
17. **Gall, J. G., M. Bellini, Z. Wu, and C. Murphy.** 1999. Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. Mol. Biol. Cell **10:**4385–4402.
18. **Gall, J. G.** 2000. Cajal bodies: the first 100 years. Annu. Rev. Cell Dev. Biol. **16:**273–300.
19. **Harris, M. E., R. Bohni, M. H. Schneiderman, L. Ramamurthy, D. Schumperli, and W. F. Marzluff.** 1991. Regulation of histone mRNA in the unperturbed cell cycle: evidence suggesting control at two posttranscriptional steps. Mol. Cell. Biol. **11:**2416–2424.
20. **Hebert, M. D., P. W. Szymczyk, K. B. Shpargel, and A. G. Matera.** 2001. Coilin forms the bridge between Cajal bodies and SMN, the spinal muscular atrophy protein. Genes Dev. **15:**2720–2729.
21. **Heintz, N.** 1991. The regulation of histone gene expression during the cell cycle. Biochim. Biophys. Acta **1088:**327–339.
22. **Leng, X., L. Connell-Crowley, D. Goodrich, and J. W. Harper.** 1997. S-phase entry upon ectopic expression of G1 cyclin-dependent kinases in the absence of retinoblastoma protein phosphorylation. Curr. Biol. **7:**709–712.
23. **Liu, J., M. D. Hebert, Y. Ye, D. J. Templeton, H. Kung, and A. G. Matera.** 2000. Cell cycle-dependent localization of the CDK2-cyclin E complex in Cajal (coiled) bodies. J. Cell Sci. **113:**1543–1552.
24. **Lukas, J., T. Herzinger, K. Hansen, M. C. Moroni, D. Resnitzky, K. Helin, S. I. Reed, and J. Bartek.** 1997. Cyclin E-induced S phase without activation of the pRb/E2F pathway. Genes Dev. **11:**1479–1492.
25. **Ma, T., N. Zou, B. Y. Lin, L. T. Chow, and J. W. Harper.** 1999. Interaction between cyclin-dependent kinases and human papillomavirus replication-initiation protein E1 is required for efficient viral replication. Proc. Natl. Acad. Sci. USA **96:**382–387.
26. **Ma, T., Van B. Tine, Y. Wei, M. Garrett, D. Nelson, P. D. Adams, J. Wang, J. Qin, L. T. Chow, and J. W. Harper.** 2000. Cell-cycle regulated phosphorylation of p220NPAT by cyclin E/Cdk2 in Cajal bodies promotes histone gene transcription. Genes Dev. **14:**2298–2313.
27. **Martin, L. G., G. W. Demers, and D. A. Galloway.** 1998. Disruption of the G_1/S transition in human papillomavirus type 16 E7-expressing human cells is associated with altered regulation of cyclin E. J. Virol. **72:**975–985.
28. **Martinelli, R., and N. Heintz.** 1994. H1TF2A, the large subunit of a heterodimeric, glutamine-rich CCAAT-binding transcription factor involved in histone H1 cell cycle regulation. Mol. Cell. Biol. **14:**8322–8332.
29. **Marzluff, W. F., and R. J. Duronio.** 2002. Histone mRNA expression: multiple levels of cell cycle regulation and important developmental consequences. Curr. Opin. Cell Biol. **14:**692–699.
30. **Marzluff, W. F., P. Gongidi, K. R. Woods, J. Jin, and L. J. Maltais.** 2002. The human and mouse replication-dependent histone genes. Genomics **80:**487–498.
31. **Matera, A. G.** 1999. RNA splicing: more clues from spinal muscular atrophy. Curr. Biol. **9:**R140–R142.
32. **Metzger, D., J. Clifford, H. Chiba, and P. Chambon.** 1995. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc. Natl. Acad. Sci. USA **92:**6991–6995.
33. **Minella, A. C., J. Swanger, E. Bryant, M. Welcker, H. Hwang, and B. E. Clurman.** 2002. p53 and p21 form an inducible barrier that protects cells against cyclin E-cdk2 deregulation. Curr. Biol. **12:**1817–1827.
34. **Nelson, D. M., X. Ye, C. Hall, H. Santos, T. Ma, G. D. Kao, T. J. Yen, J. W. Harper, and P. D. Adams.** 2002. Coupling of DNA synthesis and histone synthesis in S phase independent of cyclin/cdk2 activity. Mol. Cell. Biol. **22:**7459–7472.
35. **Nevins, J. R.** 1998. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ. **9:**585–593.
36. **Ogg, S. C., and A. I. Lamond.** 2002. Cajal bodies and coilin—moving towards function. J. Cell Biol. **159:**17–21.
37. **Platani, M., I. Goldberg, J. R. Swedlow, and A. I. Lamond.** 2000. In vivo analysis of Cajal body movement, separation, and joining in live human cells. J. Cell Biol. **151:**1561–1574.
38. **Ramsey-Ewing, A., A. J. Van Wijnen, G. S. Stein, and J. L. Stein.** 1994. Delineation of a human histone H4 cell cycle element in vivo: the master switch for H4 gene transcription. Proc. Natl. Acad. Sci. USA **91:**4475–4479.
39. **Ruesch, M. N., and L. A. Laimins.** 1997. Initiation of DNA synthesis by human papillomavirus E7 oncoproteins is resistant to p21-mediated inhibition of cyclin E-cdk2 activity. J. Virol. **71:**5570–5578.
40. **Sanchez, R., and W. F. Marzluff.** 2002. The stem-loop binding protein is required for efficient translation of histone mRNA in vivo and in vitro. Mol. Cell. Biol. **22:**7093–7104.
41. **Sherr, C. J.** 1996. Cancer cell cycles. Science **274:**1672–1677.
42. **Sherr, C. J., and J. M. Roberts.** 1999. CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev. **13:**1501–1512.
43. **Shpargel, K. B., J. K. Ospina, K. E. Tucker, A. G. Matera, and M. D. Hebert.** 2003. Control of Cajal body number is mediated by the coilin C terminus. J. Cell Sci. **116:**303–312.
44. **Spruck, C. H., K. A. Won, and S. I. Reed.** 1999. Deregulated cyclin E induces chromosome instability. Nature **401:**297–300.
45. **Trimarchi, J. M., and J. A. Lees.** 2002. Sibling rivalry in the E2F family. Nat. Rev. Mol. Cell. Biol. **3:**11–20.
46. **Tucker, K. E., M. T. Berciano, E. Y. Jacobs, D. F. LePage, K. B. Shpargel, J. J. Rossire, E. K. Chan, M. Lafarga, R. A. Conlon, and A. G. Matera.** 2001. Residual Cajal bodies in coilin knockout mice fail to recruit Sm snRNPs and SMN, the spinal muscular atrophy gene product. J. Cell Biol. **154:**293–307.
47. **van der Meijden, C. M., P. S. Vaughan, A. Staal, W. Albig, D. Doenecke, J. L. Stein, G. S. Stein, and A. J. van Wijnen.** 1998. Selective expression

of specific histone H4 genes reflects distinctions in transcription factor interactions with divergent H4 promoter elements. Biochim. Biophys. Acta **1442**:82–100.

48. **Vaughan, P. S., C. M. van der Meijden, F. Aziz, H. Harada, T. Taniguchi, A. J. van Wijnen, J. L. Stein, and G. S. Stein.** 1998. Cell cycle regulation of histone H4 gene transcription requires the oncogenic factor IRF-2. J. Biol. Chem. **273**:194–199.
49. **Vaughan, P. S., F. Aziz, A. J. van Wijnen, S. Wu, H. Harada, T. Taniguchi, K. J. Soprano, J. L. Stein, and G. S. Stein.** 1995. Activation of a cell-cycle-regulated histone gene by the oncogenic transcription factor IRF-2. Nature **377**:362–365.
50. **Wei, Y., J. Jin, and J. W. Harper.** 2003. The cyclin E/Cdk2 substrate and Cajal body component p220^{NPAT} activates histone transcription through a novel LisH-like domain. Mol. Cell. Biol. **23**:3669–3680.
51. **Whitfield, M. L., L. X. Zheng, A. Baldwin, T. Ohta, M. M. Hurt, and W. F. Marzluff.** 2000. Stem-loop binding protein, the protein that binds the 3′ end of histone mRNA, is cell cycle regulated by both translational and posttranslational mechanisms. Mol. Cell. Biol. **20**:4188–4198.
52. **Zhao, J., B. D. Dynlacht, T. Imai, T. Hori, and E. Harlow.** 1998. Expression of NPAT, a novel substrate of cyclin E-CDK2, promotes S-phase entry. Genes Dev. **12**:456–461.
53. **Zhao, J., B. K. Kennedy, B. D. Lawrence, D. Barbie, A. G. Matera, J. A. Fletcher, and E. Harlow.** 2000. NPAT links cyclin E-cdk2 to the regulation of replication-dependent histone gene transcription. Genes Dev. **14**:2283–2297.